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Dengue virus (DENV) causes pathologies ranging from the febrile illness dengue fever to the potentially lethal severe dengue disease. A major risk factor for developing severe dengue disease is the presence of subprotective DENV-reactive Abs from a previous infection (or from an immune mother), which can induce Ab-dependent enhancement of infection (ADE). However, infection in the presence of subprotective anti–DENV Abs does not always result in severe disease, suggesting that other factors influence disease severity. In this study we investigated how CD8+ T cell responses influence the outcome of Ab-mediated severe dengue disease. Mice were primed with aluminum hydroxide-adjuvanted UV-inactivated DENV prior to challenge with DENV. Priming failed to induce robust CD8+ T cell responses, and it induced nonneutralizing Ab responses that increased disease severity upon infection. Transfer of exogenous DENV-activated CD8+ T cells into primed mice prior to infection prevented Ab-dependent enhancement and dramatically reduced viral load. Our results suggest that in the presence of subprotective anti–DENV Abs, efficient CD8+ T cell responses reduce the risk of Ab-mediated severe dengue disease. The Journal of Immunology, 2014, 193: 000–000.

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Dengue virus (DENV) is the most prevalent mosquito-borne virus that affects humans (1). The four dengue serotypes (DENV1–4) can cause a spectrum of disease ranging from the self-limiting flu-like illness dengue fever to the potentially lethal severe dengue disease, during which severe bleeding, organ dysfunction, increased vascular permeability, and shock can occur (2, 3). DENV is endemic in >100 countries in tropical and subtropical regions where 2.5–3.6 billion people are at risk for infection (4, 5). An estimated 390 million cases of DENV infection occur annually, 96 million of which are apparent, 500,000 severe, and 20,000 fatal (5, 6).

A major risk factor for developing severe dengue disease is the presence of DENV-reactive Abs from a previous infection with a different serotype (heterotypic infection) or, in the case of infants, acquired from an immune mother (1, 7). This epidemiological observation led to the concept of Ab-dependent enhancement of infection (ADE), which proposes that subprotective levels of DENV-specific Abs can amplify viral infection and exacerbate disease (8, 9). However, not all secondary heterotypic infections result in severe disease (1). This suggests that although subprotective Abs can increase disease severity, other factors may also influence disease outcome.

In vitro, numerous studies have shown increased infection due to the presence of subneutralizing levels of anti–DENV Abs (10–14). In nonhuman primates, increased viremia has been demonstrated after transfer of Abs (15, 16) and, in mice, passive transfer of subprotective amounts of Abs turned a mild illness into severe dengue-like disease (17, 18). However, at present, there is no report of severe dengue disease being experimentally induced in vivo by priming with an inactivated virus that, in turn, generates disease-enhancing Ab responses directly in the primed host.

In the present study, we investigated the effects of priming with aluminum hydroxide (alum)–adjuvanted UV-inactivated DENV serotype 2 (al-UV-DENV2) on subsequent infection with DENV. Priming of mice with al-UV-DENV2 increased the severity of dengue disease via ADE upon challenge with DENV2. Priming with al-UV-DENV2 induced nonneutralizing Abs and failed to induce CD8+ T cell responses. Transfer of exogenous DENV-primed CD8+ T cells into al-UV-DENV2–primed mice prior to challenge with DENV2 prevented disease enhancement and reduced viral load, revealing that CD8+ T cells can modulate the severity of ADE-mediated dengue disease. This suggests that in the presence of subprotective levels of anti–DENV Abs, inefficient CD8+ T cell responses may further increase the risk of developing severe dengue disease, whereas efficient cellular responses reduce disease severity. Our study suggests that the quality of the CD8+ T cell response could be one of the factors that influence disease outcome when infection with DENV occurs in the presence of subneutralizing levels of Ab.

Materials and Methods

Mice

129/Sv mice deficient in type I and II IFN receptors (AG129) and wild-type 129/Sv mice were housed under specific pathogen-free conditions at the La Jolla Institute for Allergy and Immunology. Sex-matched 5- to 6-week-old mice were used. For survival studies, mice were sacrificed when moribund or at the first signs of paralysis. This study was carried out in accordance with all applicable guidelines and regulations.
with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, the Public Health Service’s Policy on Humane Care and Use of Laboratory Animals, and the Association for Assessment and Accreditation of Laboratory Animal Care International. All experimental procedures were approved and performed according to the guidelines set by the La Jolla Institute for Allergy and Immunology Animal Care and Use Committee (protocol no. AP-28SS1-0809).

**Virus production**

DENV2 strain S221 (19) was amplified in C6/36 cells and quantified by real-time quantitative RT-PCR (qRT-PCR) as previously described (20). Viral stocks were resuspended in PBS containing 10% FCS. On baby hamster kidney cell plaque assay (21), there are ~5 × 10^4 genomic equivalents (GE) per PFU for S221.

**UV inactivation**

In a well of a 12-well plate, 1 × 10^12 GE DENV2 S221 were UV-irradiated in 500 μL PBS with 500,000 μL in a UV Stratalinker 1800 (Stratagene). Subsequently, 500 μL PBS containing 10% FCS (Gemini Bioproducts) was added to reach a volume of 1 ml, to which 1 μl alum (Inject Alum, Thermo Scientific) was added dropwise while vortexing. The UV-inactivated virus/alum mix was vortexed for another 30 min at room temperature.

**Priminng and infection of mice**

Mice were injected i.p. twice (days ~14 and ~5) with 1 × 10^11 GE UV-inactivated DENV strain S221 in alum (al-UV-DENV2 in the text) in a volume of 200 μL per injection. For challenge with replicating virus, mice were infected i.v. with 5 × 10^8 GE DENV strain S221 (DENV2) diluted in 200 μL PBS with 10% FCS.

**Abs**

2H2 is an IgG2a reactive for the prM/M protein of DENV serotypes 1–4. 4G2 is a DENV-neutralizing IgG2a reactive for the E protein of DENV serotypes 1–4. 2H2 and 4G2 were produced as previously described (22). C1.18, an IgG2a isotype control of irrelevant specificity, was purchased from Bio X Cell.

**Passive Ab and serum transfer**

Abs (2H2, anti-DENV, and C1.18, isotype control) were administered i.p. in 200 μL PBS 30 min prior to infection. For serum transfer experiments, 200 μL serum was injected i.v. 1 d prior to challenge with DENV2.

**Purification of IgG from serum**

IgG was isolated from serum by protein G affinity chromatography using the NAb protein G spin kit from Thermo Scientific according to the manufacturer’s instructions. After purification, the Ab was dialyzed against PBS, sterile filtered, and the concentration was adjusted to an IgG concentration similar to the one found in the serum. DENV reactivity of the purified IgG was confirmed by DENV ELISA, and the purity was assessed by SDS-PAGE. IgG was administered i.p. in a total volume of 200 μL PBS 30 min prior to infection.

**DENV-specific IgG detection by ELISA**

DENV-reactive IgGs were detected by ELISA on DENV S221–coated plates as previously described (22). Briefly, 96-well plates were coated with sucrose-purified DENV2 strain S221 (10^7 GE/well in 50 μL 0.1 M NaHCO3); the plated virus was UV inactivated and the plates were incubated overnight at 4°C. Subsequently, plates were blocked with 100 μL Blocker Casein in PBS (Thermo Scientific, 1 h, room temperature). Blocking solution was flicked off and serum was titrated 1:3 during seven titration steps, starting with an initial dilution of 1:30 in PBS. Serum dilutions were incubated 1.5 h at room temperature, followed by washing of the plates three times with 0.05% (v/v) Tween 20 (Sigma-Aldrich) in PBS (Life Technologies). Bound Ab was detected using a 1:5000 dilution of HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) and tetramethylbenzidine (eBioscience). Results are reported as a plot of absorbance (OD 450 nm) versus dilution.

**Neutralization activity of serum**

Anti–DENV neutralizing Abs were quantified by a 50% plaque reduction neutralization test (PRNT50) as previously described (22). Serum was serially diluted 1:2, with the starting dilution being 1:10. The highest dilution reducing >50% of the plaques is reported as “50% neutralizing titer.”

**Viral RNA quantification in organs**

Viral RNA was quantified in organs by real-time qRT-PCR as described previously (20).

**Flow cytometry and intracellular cytokine staining**

Flow cytometry and intracellular cytokine staining were performed as previously described (19) with the following modifications: 2 × 10^6 splenocytes were plated for intracellular cytokine staining, and incubation time with peptide was 6 h. Peptide NS4B_99–107 (19) was used for restimulation. The following mAbs were used: anti–CD8α-PerCP-Cy5.5 (eBioscience, clone 53–6.7), anti–CD110a-PE (eBioscience, clone ID4B), anti–CD62L-Alexa Fluor 700 (eBioscience, clone MEL-14), and anti–CD4–PE-Cy7 (BD Pharmingen, clone IM7). Samples were acquired on an LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star).

**CD8+ T cell isolation and adoptive transfer**

CD8+ T cells were isolated by MACS using positive selection CD8a (Ly-2) beads from Millenyi Biotec according to the manufacturer’s instructions. After isolation, the purity of the CD8+ T cell population was assessed by flow cytometry before injection into recipient mice; our preparation contained 94.9% CD8+ T cells (data not shown). A total of 5 × 10^7 CD8+ T cells were injected i.v. into each recipient mouse in a total volume of 200 μL PBS.

**In vivo cytotoxicity assay**

AG129 recipient mice were infected with 5 × 10^7 GE S221. Eight days later, donor splenocytes were obtained from naive AG129 mice. RBCs were lysed (RBC lysis buffer, eBioscience) and cells were resuspended at 1 × 10^8 cells/ml in RPMI 1640 containing 2% FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml) (Life Technologies). Cells were pulsed with 0.5 μg/ml DENV peptide NS4B_99–107 (19) or no peptide (1 h, 37°C). Cells were washed three times and labeled with CFSE (CellTrace, Invitrogen/Molecular Probes) in PBS/0.1% BSA for 10 min at 37°C. DENV peptide-pulsed cells (target cells) were labeled with 1 μM CFSE (CFSE2); the nonlabeled cells (marker cells) with 100 μM CFSE (CFSE*). After washing excess CFSE, the two cell populations were mixed at a 1:1 ratio and 6.5 × 10^6 cells from each population were injected i.v. into naive or infected recipient mice. After 15 h, the ratio of target (CFSE2) to marker (CFSE*) cells was determined in the spleen of recipient mice by flow cytometry. Cytolytic activity in the recipient is expected to reduce the peptide-labeled target population, but not the unlabeled marker population.

**Statistical analysis**

The p values were calculated with Prism (GraphPad Software) using the unpaired t test with Welch’s correction, except for survival curves, for which the log-rank test was used. A p value < 0.05 was considered significant.

**Results**

**Priming with al-UV-DENV2 induces nonneutralizing DENV-specific IgG**

In this study, 129/Sv mice lacking type I and II IFN receptors (AG129) were used, as DENV replicates in these mice and causes a disease that recapitulates many features of DENV infection in humans (19, 23, 24). AG129 mice were primed twice (on days ~14 and ~5) with 1 × 10^11 GE al-UV-DENV2 i.p. Importantly, note that this priming regimen was not chosen to emulate vaccination schedules used in clinical settings, where the goal is to maximize the neutralizing Ab response. Our day ~14/day ~5 rapid priming schedule was chosen because, based on observations in our laboratory, it would result in the production of DENV-specific binding Abs, but not neutralizing Abs. We reasoned that those conditions would be likely to induce Ab-mediated disease enhancement (14, 18), which was necessary for our study. DENV2 strain S221 (19) was UV-inactivated to obtain a nonreplicating Ag, and alum was chosen as adjuvant because it promotes efficient Ab responses (25, 26). Analysis of the humoral immune response induced by al-UV-DENV2 shows that on day ~1, anti–DENV2 IgGs were present in the serum, as measured by ELISA (Fig. 1A), but neutralizing Ab responses were undetectable by PRNT50 (Fig. 1B).
Priming with al-UV-DENV2 increases viral load and reduces survival time upon challenge with DENV2

Next, we challenged al-UV-DENV2–primed AG129 mice with 5 × 10^8 GE DENV2 strain S221 on day 0. Two nonprimed control groups were included: 1) one group challenged with 5 × 10^8 GE DENV2 in the presence of 15 μg anti–DENV mAb 2H2, an amount that causes Ab-mediated severe dengue disease, shorter survival time, and elevated liver viral RNA titer (ADE group) (22); and 2) one group challenged with 5 × 10^8 GE DENV2 in the presence of 15 μg C1.18, an isotype control Ab of irrelevant specificity (isotype group). Abs were administered i.p. 30 min before infection. Compared to the nonprimed isotype group, al-UV-DENV2–primed mice had a reduced survival time, similar to the survival time observed in the nonprimed ADE group (Fig. 2A). On day 3, compared with the nonprimed isotype mice, al-UV-DENV2–primed mice had elevated liver viral RNA titers that were similar to the ones observed in the nonprimed ADE mice (Fig. 2B). Liver was chosen because liver viral RNA titer on day 3 correlates with disease severity (18, 22). Next, we wanted to exclude that alum or the FCS/PBS (10%) in which virus was diluted (see Materials and Methods) caused the increase in viral RNA observed in the liver 3 d after challenge. Therefore, AG129 mice were primed on days −14 and −5 with either al-UV-DENV2 (as previously) or with FCS/PBS (10%) in alum without virus (vehicle only). On day 0, mice were challenged with DENV2, and viral RNA levels were determined in the liver on day 3. As a control, one group was left untreated until challenge on day 0. Compared to nonprimed controls, viral RNA levels were elevated in the al-UV-DENV2–primed mice, but not in the mice primed with alum and FCS/PBS without virus (Fig. 2C). This result demonstrates that the presence of UV-inactivated virus was required in the alum preparation to induce the viral titer increase observed in the liver on day 3 after challenge with DENV2. Therefore, we excluded a nonspecific effect of the vehicle itself. In summary, priming with al-UV-DENV2 induced nonneutralizing anti–DENV Abs and resulted in reduced survival time and increased viral load upon DENV2 challenge.

Passive transfer of al-UV-DENV2 immune serum increases viral titer in the liver upon challenge with DENV2

To demonstrate that Ab induced by al-UV-DENV2 priming was responsible for the disease enhancement observed above, 200 μl serum from al-UV-DENV2–primed AG129 mice were transferred i.v. into naive AG129 mice 1 d before challenge with DENV2. Two control groups that did not receive immune serum were included: the ADE and isotype groups as described above. Compared to the isotype group with no serum transfer, mice that had received al-UV-DENV2 immune serum prior to challenge had elevated liver viral RNA levels 3 d after challenge (Fig. 3A). This suggests that the Abs present in the al-UV-DENV2 immune serum were responsible for the increase in viral RNA levels observed upon challenge in al-UV-DENV2–primed AG129 mice.

To confirm that the enhancement caused by passive transfer of al-UV-DENV2 immune serum from AG129 mice (lacking type I and II IFN receptors) would also occur after transfer of wild-type (WT) immune serum, WT mice were primed with al-UV-DENV2 and serum was collected on day 0. As observed with AG129 mice, nonneutralizing DENV2-specific IgGs were detected in the serum of al-UV-DENV2–primed WT mice (Supplemental Fig. 1). Transfer of al-UV-DENV2 immune WT serum into naive AG129 mice prior to challenge with DENV2 increased liver viral RNA levels by ∼10-fold 3 d postinfection (Fig. 3B).

To exclude a role for complement proteins in the enhancement observed after serum transfer, another experiment was performed where al-UV-DENV2 immune serum from WT mice was heat inactivated (56˚C, 30 min) prior to transfer into AG129 recipients. Additionally, to verify that the IgG present in the transferred serum was responsible for the increase in DENV infection, IgG was purified out of al-UV-DENV2 immune serum by protein G affinity chromatography and was transferred into naive recipients prior to challenge with DENV2. The amount of purified IgG transferred was equivalent to the amount of IgG present in the 200 μl serum transferred in previous experiments. The DENV reactivity of the isolated IgG was confirmed by ELISA, and its purity was confirmed by SDS-PAGE (Supplemental Fig. 2). As shown in Fig. 3C, transfer of either untreated or heat-inactivated al-UV-DENV2 immune serum resulted in higher DENV titers in the recipients upon challenge, as did transfer of purified IgG from UV-DENV2 serum. These results confirm that priming with al-UV-DENV2 induced an Ab response that, in turn, caused Ab-mediated disease enhancement upon challenge with DENV2.

Priming with al-UV-DENV2 does not activate CD8+ T cells

Next, we hypothesized that priming with al-UV-DENV2 resulted in severe disease because of the simultaneous presence of nonneutralizing anti–DENV Abs and inefficient CD8+ T cell responses. We expected CD8+ T cell responses to be low and/or inefficient after priming with al-UV-DENV2 because 1) alum skew immune responses toward humoral rather than cellular immunity (25), 2) efficient CD8+ T cell responses are not readily induced by nonreplicating Ags (27), and 3) priming with al-UV-DENV2, which is nonreplicating, does not result in production of nonstructural proteins, which are the main targets of anti–DENV CD8+ T cell responses (19, 28, 29). Thus, the number and phenotype...
of CD8+ T cells were assessed in the spleen of AG129 mice that were either primed with al-UV-DENV2 on days −14 and −5 or infected with $5 \times 10^5$ GE DENV2 on day −8 (Fig. 4). Compared to naive animals, the total number and percentage of CD8+ T cells increased in the spleen of animals infected with replicating DENV2, but not in the spleen of animals primed with non-replicating al-UV-DENV2 (Fig. 4A, 4B). Similarly, >50% of the CD8+ T cells were activated (CD44hiCD62Llo) after infection with
DENV2, but the percentage of activated CD8+ T cells in al-UV-DENV2–primed mice was comparable to the percentage in naive mice (Fig. 4C). These results demonstrate that priming with al-UV-DENV2 failed to induce measurable CD8+ T cell responses.

Transfer of DENV-primed CD8+ T cells in al-UV-DENV2–primed mice prevents Ab-mediated disease enhancement and reduces viral load upon challenge with DENV2

We hypothesized that transferring CD8+ T cells from an animal that had been infected with (replicating) DENV2 into al-UV-DENV2–primed recipients prior to challenge would prevent the Ab-induced increase in viral load. First, we verified that priming with al-UV-DENV2 failed to induce measurable CD8+ T cell responses.

We hypothesized that transferring CD8+ T cells from an animal that had been infected with (replicating) DENV2 into al-UV-DENV2–primed recipients prior to challenge would prevent the Ab-induced increase in viral load. First, we verified that priming with al-UV-DENV2 failed to induce measurable CD8+ T cell responses. AG129 mice were infected with DENV2 and 8 d later splenocytes were restimulated with NS4B 99–107, an immunodominant DENV peptide from the NS4B protein (19). After in vitro restimulation, CD107a+ (degranulation marker) CD8+ T cells were present in the DENV2-infected animals, but not in naive mice (Fig. 5A). In another experiment, the cytolytic activity of the T cells was assessed in vivo cytotoxicity assay. Equal numbers of naive splenocytes labeled with the DENV peptide NS4B 99–107 (CFSEhi target cells) and unlabeled splenocytes (CFSElo marker cells) were transferred in naive or infected recipient mice [infection as in (A)]. The target/marker ratio was determined by FACS in the spleen of recipient mice 15 h after transfer. (C) AG129 mice were primed with al-UV-DENV2 on days −14 and −5 (or not primed) and challenged on day 0 with 5 × 10^6 GE DENV2 i.v. On day −1, some mice received 5 × 10^7 CD8+ T cells from mice infected with 5 × 10^8 GE DENV2 seven days earlier. Viral RNA titers were measured on day 3 postchallenge in the liver. For (A)–(C), each symbol represents one mouse; n = 4–6; experiment (A) was repeated twice with similar results. Samples with DENV RNA content under the detection limit are depicted in gray and have no numerical value. For statistical analysis, the value of the detection limit was attributed to samples under the detection limit to calculate the p value. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

FIGURE 4. Priming with al-UV-DENV2 does not induce measurable CD8+ T cell responses. AG129 mice were primed with al-UV-DENV2 on days −14 and −5 or infected with DENV2 on day −8. On day 0, the total number (A) and the percentage (B) of CD8+ T cells were determined in the spleen, and the percentage of activated cells (CD44hiCD62Llo) within the CD8+ T cell population was determined (C) by flow cytometry. Two independent experiments were pooled; n = 8 total. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

FIGURE 5. Transfer of DENV-primed CD8+ T cells prevents ADE and reduces viral load after al-UV-DENV2 priming. (A) AG129 mice were infected with 5 × 10^8 GE DENV2 and 8 d later the percentage of CD8+ T cells staining positive for CD107a (marker of degranulation) was determined by FACS after in vitro restimulation with the DENV peptide NS4B 99–107. (B) The functionality of DENV2-primed CD8+ T cells was assessed by in vivo cytotoxicity assay: equal numbers of naive splenocytes labeled with the DENV peptide NS4B 99–107 (CFSEhi target cells) and unlabeled splenocytes (CFSElo marker cells) were transferred in naive or infected recipient mice [infection as in (A)]. The target/marker ratio was determined by FACS in the spleen of recipient mice 15 h after transfer. (C) AG129 mice were primed with al-UV-DENV2 on days −14 and −5 (or not primed) and challenged on day 0 with 5 × 10^6 GE DENV2 i.v. On day −1, some mice received 5 × 10^7 CD8+ T cells from mice infected with 5 × 10^8 GE DENV2 seven days earlier. Viral RNA titers were measured on day 3 postchallenge in the liver. For (A)–(C), each symbol represents one mouse; n = 4–6; experiment (A) was repeated twice with similar results. Samples with DENV RNA content under the detection limit are depicted in gray and have no numerical value. For statistical analysis, the value of the detection limit was attributed to samples under the detection limit to calculate the p value. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
peptide NS4B_99–107 (CFSEhi target cells) and splenocytes without peptide (CFSElo marker cells) were transferred into naïve or DENV2-infected recipient mice 8 d postinfection. The target/marker ratio was determined by FACS in the spleen of recipient mice 15 h after transfer. Cytotoxic CD8+ T cells are expected to lyse peptide-labeled target cells, but not marker cells, thereby reducing the target/marker ratio. Fig. 5B shows that cytolytic activity was detected in DENV2-infected mice but not in naïve mice. Therefore, we concluded that DENV-specific CD8+ T cells from AG129 mice were functional and capable of lysing target cells after infection with DENV2.

Next, we tested whether transfer of CD8+ T cells from DENV2-infected donors into al-UV-DENV2–primed recipients would prevent Ab-mediated increase in viral load. To this end, 5 × 10⁷ CD8+ T cells (positive selection of CD8+ T cells) from day 7 DENV2-infected AG129 mice were adoptively transferred into al-UV-DENV2–primed AG129 mice 1 d prior to DENV2 challenge. As a control, one group of al-UV-DENV2–primed mice did not receive CD8+ T cells. Two groups of nonprimed mice (with and without transferred CD8+ T cells) were included. When no CD8+ T cells were transferred, viral RNA levels in the liver 3 d after challenge were higher in al-UV-DENV2–primed animals relative to nonprimed animals (Fig. 5C). Viral RNA was undetectable in nonprimed animals in which CD8+ T cells had been transferred. Similarly, no viral RNA was detected in al-UV-DENV2–primed animals that had received CD8+ T cells prior to challenge.

These results demonstrate that transfer of DENV2-primed CD8+ T cells prior to challenge with DENV2 dramatically reduced viral load in both nonprimed and al-UV-DENV2–primed mice. Similar results were obtained in an experiment where total T cells (negative selection of untouched total T cells) from DENV2-infected animals were transferred between al-UV-DENV2 priming and DENV2 challenge (data not shown). The results of our study are summarized in Fig. 6.

**Discussion**

In this study, we investigated the effect of priming with al-UV-DENV2 on subsequent infection with DENV2 in the absence or presence of exogenous DENV2-primed CD8+ T cells. Priming of type I and II IFN receptor–deficient AG129 mice with al-UV-DENV2 reduced survival time and increased liver viral load upon subsequent infection with DENV2. Priming with nonreplicating al-UV-DENV2 induced nonneutralizing DENV2-specific IgG, which in turn mediated ADE upon challenge with (replicating) DENV2 (Fig. 6). To our knowledge, the present study demonstrates for the first time that priming with an inactivated virus can elicit an Ab response that can induce Ab-mediated dengue disease enhancement upon challenge. Although ADE following passive transfer of immune serum or mAbs has been widely demonstrated in animal models (15, 17, 18, 22, 30, 31), our study describes a direct phenomenon in which priming with an inactivated virus results in ADE upon challenge of the same animal, not via serum transfer.

In contrast to infection with replicating DENV2, priming with al-UV-DENV2 did not induce measurable CD8+ T cell responses. Transfer of DENV2-primed CD8+ T cells into al-UV-DENV2–primed recipients prevented Ab-dependent enhancement of disease and reduced viral load upon DENV2 challenge (Fig. 6). Our results suggest that in the presence of enhancing amounts of Abs, efficient CD8+ T cell responses limit disease severity and ADE.

A caveat of our study is the use of mice deficient in type I and II IFN receptors (AG129). Because IFN is a key player of the immune system, we would suggest great caution when extrapolating our results to dengue disease in humans. However, we chose to use AG129 mice because DENV replicates to measurable levels and causes a dengue-like disease in these mice (32, 33). This allows for assessment of the effect of al-UV-DENV2 priming on a subsequent DENV infection. The experiments described in the present study would not be feasible in WT mice, in which DENV does not replicate. The lack of replication in WT mice may be explained by
the fact that DENV inhibits IFN signaling pathways to establish infection in humans, but is unable to do so in mice (34–37). Although using IFNAR-deficient mice is not ideal, AG129 mice have been increasingly used as a model to study DENV-induced pathology and immunity (17, 18, 22, 24, 30, 31, 38–40). Use of these mice permits us to measure dengue virus infection outcomes that may be relevant to humans.

The rapid priming schedule chosen in our study was not meant to emulate vaccination schedules used in clinical settings, which aim at obtaining the best possible protective immunity via optimal affinity maturation of the Ab response. In contrast, in our study, the priming regimen was chosen specifically to induce a non-neutralizing DENV-specific Ab response, which has the potential to induce Ab-dependent enhancement of disease. Because this priming schedule did not induce robust endogenous CD8+ T cell responses, we were able to transfer exogenous DENV-primed CD8+ T cells and investigate their impact on the outcome of Ab-induced severe dengue disease.

In humans, ADE can be observed when patients are sequentially infected with two different DENV serotypes (heterotypic secondary infections) (1, 7). However, in our study, priming with al-UV-DENV resulted in ADE upon challenge with the same serotype (homotypic challenge). The Ab response induced by priming did not reach the threshold required for neutralization. Consequently, the sub-neutralizing character of the response induced by priming was sufficient to induce ADE upon challenge, regardless of the serospecificity. This is consistent with experimental evidence demonstrating that even neutralizing Abs can induce ADE at lower concentrations (41, 42), both in vitro (13) and in vivo (18).

The low CD8+ T cell responses observed after priming with al-UV-DENV2 could be explained because the Ag was given in alum, which is known to skew the immune response toward humoral rather than cellular immunity (25, 26). Additionally, the priming virus was UV inactivated and, consequently, unable to replicate. Therefore, priming did not result in production of nonstructural viral proteins, which are the major CD8+ T cell targets during natural DENV infection (19, 28, 29). Furthermore, a nonreplicating Ag is less likely to induce robust T cell responses compared with a replicating virus (27). Transfer of T cells from mice infected with replicating DENV2 into al-UV-DENV2–primed mice prior to viral challenge dramatically reduced viral RNA levels upon infection.

Keeping in mind the limitations of our model and the pitfalls associated with extrapolating data from animal models to human disease, we speculate on some parallels between our results and what has been observed in humans. In our study, ADE seemed to be the result of the combination of subprotective, nonneutralizing anti–DENV Abs and nonexistent anti–DENV CD8+ T cell responses. In humans, although the presence of anti–DENV Abs from a previous heterotypic infection (or from an immune mother) may be a major risk factor for developing severe dengue disease, not all patients with pre-existing anti–DENV Abs develop severe disease upon heterotypic reinfection (1). In fact, only a small percentage of secondary DENV infections result in shock (1). We suggest that the risk of developing severe dengue disease is higher in patients that have both subprotective levels of Abs and inefficient (or nonexistent) T cell responses.

Our study shows that the absence of a robust CD8+ T cell response negatively influences disease outcome in the presence of subneutralizing Abs, but that does not exclude the contribution of other factors to disease outcome. Many other host and viral factors influence disease outcome, including the quality of the Ab response, the interval between infections, differences in strain virulence, and overall immune status of the host. Therefore, although many factors can influence disease outcome, our data suggest that in the presence of Ag-induced subneutralizing levels of Abs, an inefficient (or absent) CD8+ T cell response may be detrimental to the host.

In patients, the protective efficacy of T cell responses may be influenced by several factors, including the HLA phenotype. During secondary DENV infections, some alleles were associated with disease susceptibility and others were associated with protection (43, 44). Different HLA alleles were also associated with differential magnitude of cellular anti–DENV responses in humans (29). Furthermore, HLA alleles associated with increased risk of severe dengue disease (45, 46) were associated with weaker CD8+ T cell responses, whereas alleles associated with decreased risk of severe disease were associated with higher and polyfunctional cellular responses (29). These findings support the hypothesis that during secondary infections, disease outcome may be influenced by both the serological status of the host and the magnitude and/or quality of cellular immune responses.

In the case of infants born to DENV-immune mothers, DENV infection is more likely to evolve into severe dengue disease between 5 and 12 mo of age, a time during which passively transferred maternal DENV-specific Abs have dropped below protective levels but have not yet disappeared completely (1, 9, 47–51). As those infants have never been exposed to DENV, they do not have DENV-specific memory T cells. Therefore, the increased susceptibility to severe dengue disease is observed in a situation where exogenous subprotective levels of DENV-specific Abs (from the mother) are already present at the time of infection, whereas endogenous anti–DENV CD8+ T cell responses only start to be primed at the time of infection. Perhaps Abs initiate enhancement of infection before endogenous CD8+ T cell responses begin to limit viral spread. Additionally, endogenous anti–DENV cellular responses may be weak in infants, as their immune system is not yet fully functional (52).

Based on these observations and on our results, we hypothesize that in the presence of subprotective humoral immunity, inefficient (or absent) CD8+ T cell responses may be an additional risk factor for developing severe dengue disease, whereas robust cellular responses may prevent Ab-mediated severe dengue disease. Our data may contribute to explain why infection in the presence of subprotective levels of anti–DENV Abs does not always result in severe dengue disease.

Disclosures
The authors have no financial conflicts of interest.

References
Supplementary figure S1 - Priming with al-UV-DENV2 induces non-neutralizing DENV-specific IgG in WT mice (related to figure 3).

WT mice were primed with al-UV-DENV2 on days -14 and -5. On day 0, DENV-specific IgG were measured in the serum by ELISA (A) and the presence of neutralizing Abs was detected by PRNT_{50} (B).

In A, n=4 for the experimental group, one naïve sample was included as a negative control. In B, each symbol represents one mouse; two naïve samples and the DENV-neutralizing Ab 4G2 were used as controls. The dotted line represents the background signal in A, and the detection limit in B. Samples under the detection limit are represented in grey.
Supplementary figure S2 – IgG purified from al-UV-DENV2 immune serum retains DENV-specificity (related to figure 3).

WT mice were primed with al-UV-DENV2 on days -14 and -5. On day 0, serum was collected and IgG was isolated by protein G purification. After isolation, the purified IgG concentration was adjusted to match the IgG concentration in the serum. The DENV-reactivity of the purified IgG was confirmed by DENV-specific IgG ELISA (A). The purity of the IgG was confirmed by SDS-PAGE (B). 5 µl of serum or purified IgG were used for ELISA and 5 µg of reduced-protein were loaded on the gel; a commercially available monoclonal IgG was loaded on the gel as a control.